Effects *in vitro* of two marine substances, chlorolissoclimide and dichlorolissoclimide, on a non-small-cell bronchopulmonary carcinoma line (NSCLC-N6)

C. Malochet-Grivois, C. Roussakis, N. Robillard[1], J.F. Biard, D. Riou, C. Débitus[2] & J.F. Verbist

Substances Marines à Activités Biologiques, faculté de Pharmacie, B.P. 1024, 44035 Nantes Cedex 01, [1]Service Commun de Cytométrie en.Flux, Laboratoire d'Hématologie, CHR, 44035 Nantes Cedex 01, France and [2]Laboratoire de Pharmacologie, ORSTOM, B.P. A5, Nouméa, New Caledonia

Summary: The antiproliferative activity of two nitrogenous labdane cytotoxic substances from *Lissoclinum voeltzkowi* Michaelson (Urochordata), dichlorolissoclimide (P2) and chlorolissoclimide (P1), was studied *in vitro* on a continuous human non-small-cell bronchopulmonary carcinoma line (NSCLC-N6) at the cell cycle level. This antiproliferative effect resulted from a blockade of G1 phase cells. Mortality occurred, regardless of the degree of cell ploidy, with cell transition to an out-of-cycle situation characteristic of a G1D terminal maturation state.

Key words: chemosensitivity/chlorolissoclimide/dichlorolissoclimide/differentiation/ lung carcinoma/phase G1

Introduction

Chemotherapy has proved difficult for colon, lung and other solid tumours with extremely slow doubling times, ranging up to 3 months in man. As the ineffectiveness of alkylating agents has been clinically demonstrated (Williams, 1989), it is essential to find new substances with different mechanisms of action. The existence of substances capable of inducing irreversible blockade of G1 phase cells suggests that there is a mechanism of action related to induction of G1D terminal differentiation (Wille *et al.*, 1982; Wille & Scott, 1986) which could provide a new approach to the treatment of slowly developing cancers. One of the simplest methods is to study the effect of these substances at the level of the cell cycle. The purpose of the present study was to investigate the effect of chlorolissoclimide and dichlorolissoclimide on asynchronous cells of a human non-small-cell bronchopulmonary carcinoma line (NSCLC-N6). Chlorolissoclimide and dichloro-lissoclimide are substances previously identified in a New Caledonian urochordate, *Lissoclinum voeltzkowi* Michaelson (Malochet-Grivois *et al.*, 1991) which have a labdane-type diterpenic structure, with one or two chlorine(s) and a succinimide function (Figure 1).
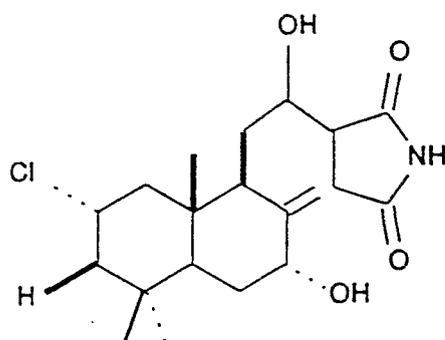
Dichlorolissoclimide

Chlorolissoclimide

Figure 1   Structure of chlorolissoclimide and dichlorolissoclimide

Materials and methods

Chlorolissoclimide (P1) and dichlorolissoclimide (P2) (respective mol. wt 383 and 417 kDa) were isolated from a New Caledonian ascidian, *L. voeltzkowi* Michaelson 1920, collected as previously described (Verbist *et al.*, 1992). The purity of the substances (99%) was determined by mass spectrometry and high-pressure liquid chromatography.

Cell line and cell culture

The NSCLC-N6 cell line (Roussakis *et al.*, 1991a) derived from a human non-small-cell bronchopulmonary carcinoma (moderately differentiated, rarely keratinizing, classified

as T2N0M0) was used for all determinations. The line was grafted into a nude mouse and then cultured in RPMI 1640 medium (Intermed) with 5% fetal calf serum, to which were added 100 IU penicillin/ml, 100 μg streptomycin/ml and 2 mM glutamine. Cells were cultured at 0.4×10^5/ml at 37°C in an air:carbon dioxide (95:5, v/v) atmosphere. In these *in vitro* conditions, cell doubling time was ~ 48 h.

Cytotoxicity determination

In continuous drug exposure.   Experiments were performed in microtitre plates (0.1×10^5 cells/ml). P1 and P2 were tested at various concentrations. Cell growth was estimated by a colorimetric assay based on conversion of tetrazolium dye (MTT) to a blue formazan product using live mitochondria (Mosmann, 1983) at 0, 5, 24, 48, 72 and 96 h. Eight determinations were performed for each concentration. Control growth (without P1 and P2) was estimated from 16 determinations. The confidence intervals in Figures 2 and 3 represent the standard deviations calculated for each of the points.

In discontinuous drug exposure.   Cells were incubated for 96 h in 75 cm² flasks at a concentration of 2×10^5 cells/ml in the culture conditions described above and in the presence or absence of 0.036 or 1 μg/ml. After medium removal, cells were washed twice with phosphate-buffered saline to eliminate drug traces and then 100 μl of cell suspension were added to a microtitre plate at concentrations of 1×10^5 cells/ml in fresh medium containing no drug. Cell growth was evaluated by the colorimetric assay of Mosmann (1983) (using MTT) on this day (considered as day 0) and on days 1, 2 and 3.

Flow cytometry assay

For DNA staining, 0.8×10^5 cells were cultured in 25 cm² flasks in the presence and absence of 0.004, 0.036 and 1 μg/ml of P1 and P2. Determinations were performed at 8, 24, 48, 72 and 96 h. As the NSCLC-N6 line has a very strong capacity for forming cell clusters, DNA staining was carried out using the technique of Vindelov (1977). Cells were stained directly in the flask. After removal of culture medium, 1 ml Vindelov solution [50.01 M glycine/NaOH, 9.6×10^5 M propidium iodide (Sigma R5250), 0.3 M NaCl, pH 10] was added to the flasks, which were shaken and then left in darkness at 4°C for 15 min. The cell suspension thus obtained was filtered on nylon mesh (50 μm) and analysed.

.   All flow cytometry experiments were performed independently eight times. The DNA content of 20 000 naked nuclei was measured by an ATC 3000 flow cytometer (Brucker, Wissembourg, France) connected to an MCA 3000 multichannel analyser (Brucker). Propidium iodide was excited (488 nm) using an argon laser (Innova 90.5 UV, Coherent Laser Products Division, Palo Alto, CA) at 400 mW. Flow resonance was measured at >610 wavelengths. To eliminate doublet nuclei for DNA area histogram analysis, the DNA peak versus the DNA area was selected by gating on the cytogram.

For flow cytometric analysis, cells not treated with P1 and P2 in G0/G1, S and G2M phases were estimated using the mathematical model of Fried & Mandel (1979). Cellular debris resulting from the action of P1 and P2 was apparent on the DNA histograms.
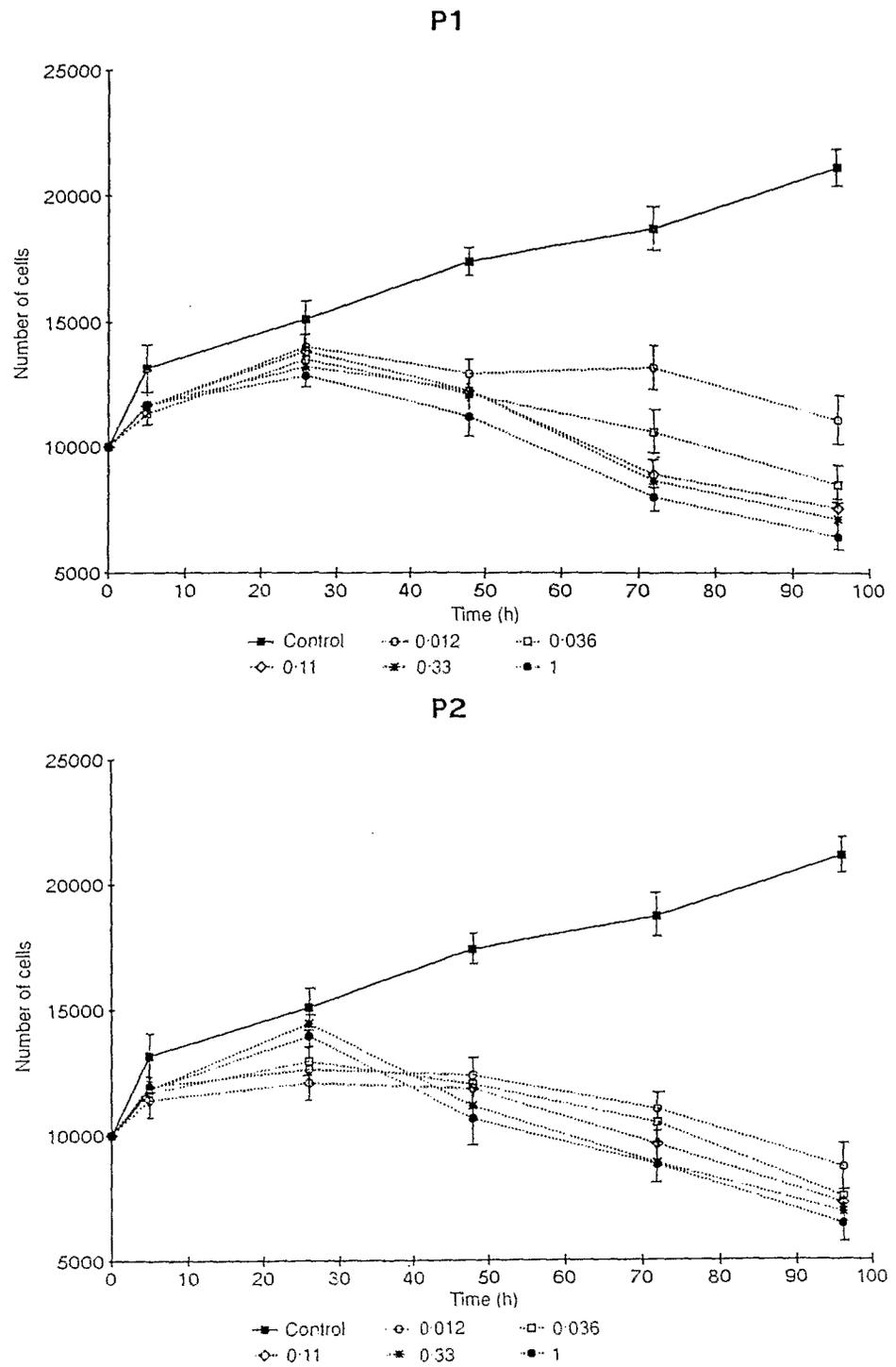
Results

Cytotoxicity

Figure 2a and b shows the growth of NSCLC-N6 cells in the presence and absence of 0.012, 0.036, 0.11, 0.33 and 1 μg/ml of P1 and P2 as determined at different time points.

P1

P2

Figure 2 (a) Growth kinetics after P1 treatment in continuous drug exposure. (b) Growth kinetics after P2 treatment in continuous drug exposure

The figure shows that there was no increase in cell numbers with either drug at any concentration when cells had been exposed for 50 h. After this time, there was an apparent decrease in 'cell numbers' in some cultures. The concentration of drug required for 50% cell destruction (IC_{50}) was 0.01 µg/ml for P1 and 0.009 µg/ml for P2 at 72 h.

Figure 3 shows the irreversibility of proliferation arrest due to P1 contact with NSCLC-N6 cells for 96 h. Moreover, inhibition of cell proliferation continued even in the absence of P1 after 96 h.
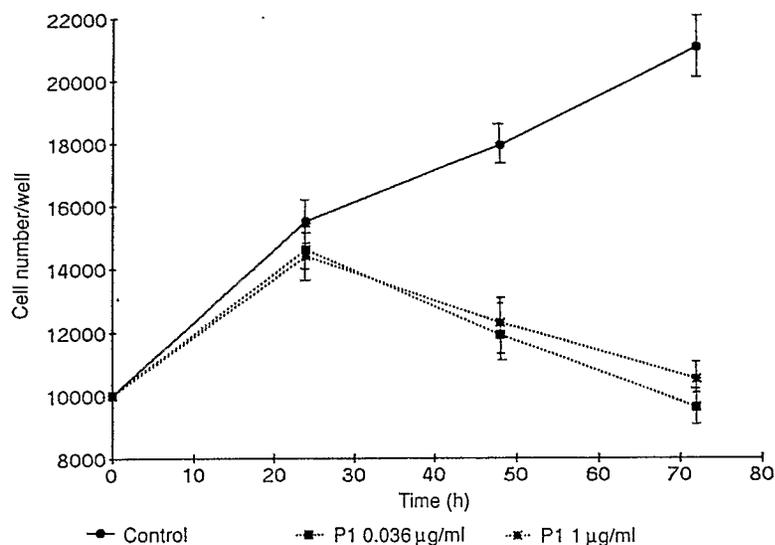

Figure 3 Growth kintics after P1 treatment in discontinuous drug exposure

Effects on the cell cycle

Figures 4 and 5 show the effect of P1 and P2 on the cell cycle as a function of concentration. A blockade of cells in the G1 phase was demonstrated by a dose-dependent increase in G1 phase and a decrease in S and G2M phases. However, the '4C' increase was noted simultaneously in G1 phase cells at 72 h, even after treatment with 0.004 µg/ml of P1 and P2.

Figures 5, 6, 7 and 8 illustrate the time-dependent effects of P1 and P2 on the cell cycle. A blockade of G1 phase cells was noted at 48 h, after treatment with 0.004 µg/ml. At 48 h, the percentage of S and G2M phase cells had decreased in a time-dependent manner. The appearance of debris, as well as the increased coefficient of variation (CV) of the '2C' peak at 48 h, could be considered to be indices of cell mortality. These effects were even more marked at 96 h.

Discussion

Chlorolissoclimide (P1) and dichlorolissoclimide (P2), when tested in a continuous drug exposure experiment, showed cytotoxicity toward cells of the NSCLC-N6 line at doses of
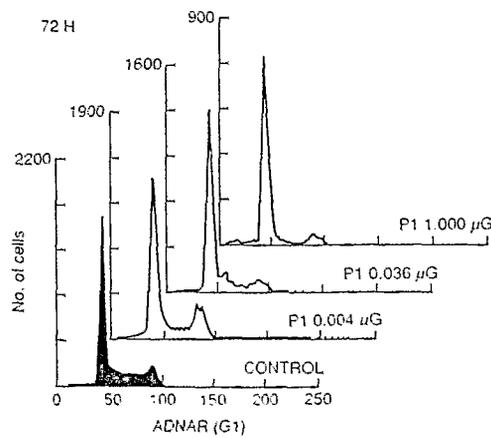
Figure 4   Histogram of the DNA of NSCLC-N6 cells cultured for 72 h in the presence of different P1 concentrations. After 72 h of culture in the presence of P1, cells were blocked in G1 phase. This blockade was dose dependent and associated with both a decrease in the number of cells in S and G2M phases, and the appearance of debris from the first 'dying' cells blocked and induced in G1D phase. At the lowest dose, the increase in the '4C' peak, which was non-dose dependent compared to controls, indicated that '4n' cells were totally blocked in G1 phase
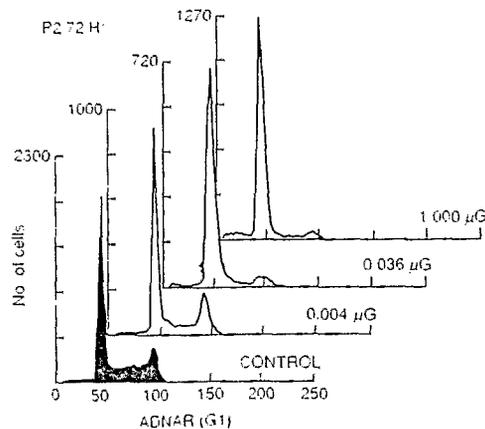

Figure 5   Histogram of the DNA of NSCLC-N6 cells cultured for 72 h in the presence of different P2 concentrations. After 72 h of culture in the presence of P2, cells were blocked in G1 phase. This blockade was dose dependent and associated with both a decrease in the number of cells in S and G2M phases, and the appearance of a greater quantity of cellular debris than that produced by P1 at the same dose. The cellular debris was also due to the first 'dying' cells blocked and induced in G1D phase. The increase in the '4C' peak at the lowest dose was non-dose dependent compared to controls, which indicated as for P1 that '4n' cells were totally blocked in G1 phase
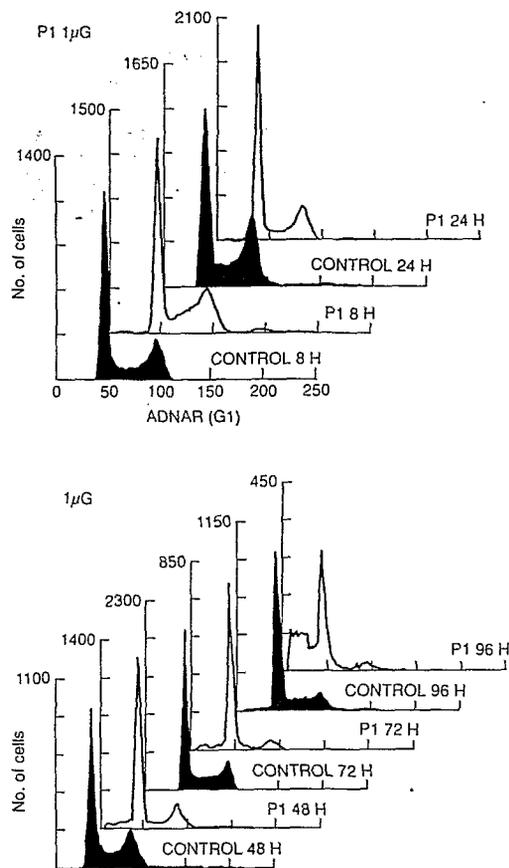
Figure 6   Effect of 1 μg/ml of P1 as a function of culture time (8, 24, 48, 72 or 96 h).
The increase in the number of G1 phase cells was time dependent and, beginning at 24
h, led to a decrease in the number of S phase cells. This effect was associated with the
presence of a considerable amount of cellular debris which was proportionally greater
as contact time increased (96 h) (d = cellular debris). The increase in the '4C' peak at 8
h, which was non-time dependent compared to controls, indicated that '4n' cells were
blocked in G1 phase

the order of a few ng/ml. To determine the nature of the IC_{50} dose at cell level, we
studied the effect of P1 and P2 on NSCLC-N6 cells as a function of time, both in
continuous and discontinuous treatment. We found a total cessation of cell proliferation
after 24 h in continuous drug exposure, as though the cells had lost their mitotic capacity
while remaining viable until at least 72 h (Figure 2). This cessation of cell proliferation
was irreversible. Cell mortality continued in the absence of P1 and P2, after 72 h of
treatment and replenishing of the nutrient medium (Figure 3). To examine these results
more thoroughly, we performed a flow cytometric study to differentiate the cells accord-
ing to their DNA content and thus their phase in the cell cycle. DNA content was
proportional to the intercalary fluorochrome intensity bound to the double strands of
DNA.

In this study of the mode of action of P1 and P2, cell mortality was indicated by the
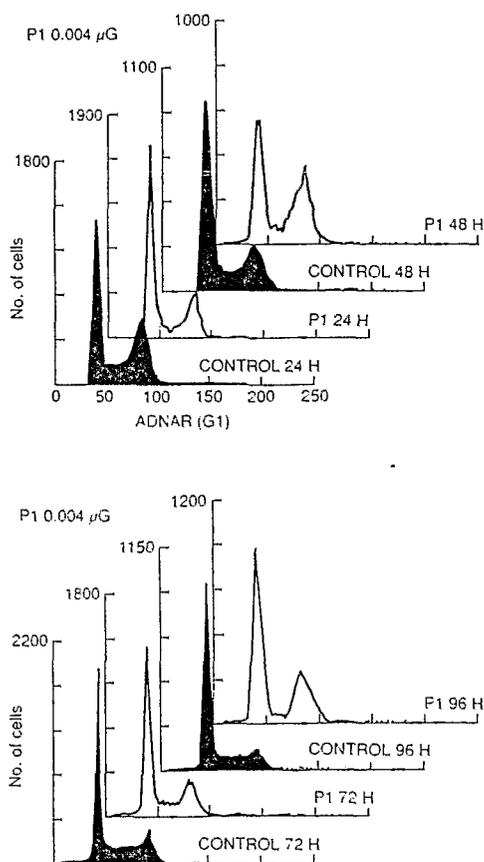
Figure 7   Effect of 0.004 μg/ml of P1 as a function of culture time (24, 48, 72 or 96 h). The increase in the number of G1 phase cells was less than with a 1 μg/ml dose of P1, although still time dependent. Beginning at 8 h, an increase was noted in the '4C' and '2C' peaks, to the detriment of the S phase as compared to controls. An increase in cellular debris was indicative of cell mortality. At 96 h, an intensification of the phenomenon indicated that the '4C' peak was due to '4*n*' cells blocked in G1 phase

presence of cellular debris upstream from the G1 phase. If this debris were eliminated by a gate on the cytogram [forward angle light scatter (FALS) versus the DNA peak or FALS versus wide angle light scatter (WALS)], an overestimation of the S phase would have been likely. It thus seemed preferable to let this debris appear, to the detriment of the DNA histogram. Elimination of the debris would have been possible by mathematical processing of the histograms, but too many data would then have been lost logarithmically and the resulting histogram would not have been reliable.

We noted that the antiproliferative effect of P1 and P2 on asynchronous cultures of the heterogeneous NSCLC-N6 cell line was due to cell blockade in G1 phase, similar to that produced by inducers of terminal differentiation (Roussakis *et al.*, 1991b; Riou *et al.*, 1992). This blockade was irreversible, total, dose- and time-dependent, and apparently concerned all treated cells. The decrease in the '4C' peak at 72 h of treatment induced by
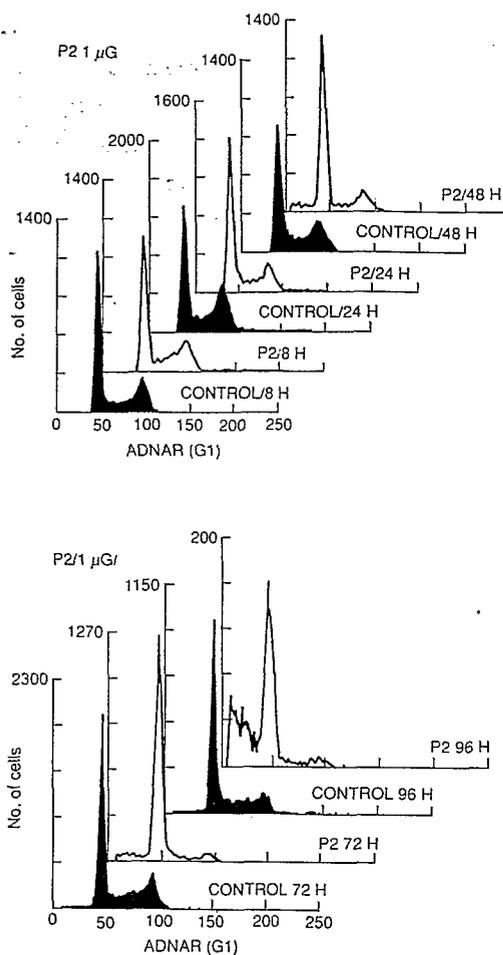
Figure 8   Effect of 1 μg/ml of P2 as a function of culture time (24, 48, 72 or 96 h). The increase in the number of G1 phase cells was time dependent and, beginning at 24 h, also led to a decrease in the number of S phase cells. This effect was associated with the presence of a considerable amount of cellular debris which was proportionally greater as contact time increased (96 h) (d = cellular debris). The increase in the '4C' peak at 8 h and the decrease in this peak at 24, 72 and 96 h, compared to controls and to the detriment of the S phase, indicated that '4n' cells were blocked in G1 phase

1 μg/ml, to the detriment of the S phase compared to 24 h, indicated that this peak corresponded to '4n' polyploid NSCLC-N6 cells blocked in G1 phase.

Substances P1 and P2 are thus capable of definitively blocking cancer cells independently of their DNA quantity. This is an interesting result since solid tumours are characterized by heterogeneity in their degree of ploidy which could have an influence on their chemosensitivity (Gratas et al., 1990).

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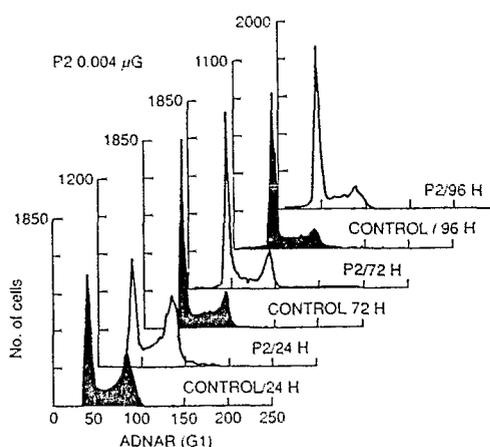it, and apparently
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Figure 9   Effect of 0.004 μg/ml of P2 as a function of culture time (24, 48, 72 or 96 h). The increase in the number of G1 phase cells was less than with a higher (1 μg/ml) dose of P1, although still time dependent. Beginning at 24 h, an increase was noted in '4C' and '2C' peaks, to the detriment of the S phase as compared to controls. An increase in cellular debris was indicative of cell mortality. At 72 h, the decrease in the '4C' peak, to the detriment of the S phase compared to 24 h, indicated that '4*n*' cells were blocked in G1 phase

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